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(54) Title: METHOD FOR DETECTING THE EXPRESSION OF BIOREGULATORY GENES IN MICROCULTURES OF EUKARYOTIC CELLS AND KIT THEREFOR

(57) Abstract

A method allowing accurate and sensitive detection of a transient expression of bioregulatory genes in microcultures of eukaryotic cells. The method comprises the steps of (a) isolating and culturing the cells under conditions inducing the expression of at least one of the said genes, (b) lysing the cells with guanidinium hydrochloride, (c) selectively precipitating ribonucleic acid by adding to the homogenised cell preparation a sodium or potassium acetate solution followed by absolute ethanol, (d) recovery and blotting RNA onto a support matrix, (e) hybridizing recovered and blotted RNA with a labelled riboprobe specific for at least one of said genes, and (f) quantitatively determining the extent of binding of the labelled riboprobe to the blotted RNA. The use of varying induction processes on separate aliquots of a cell sample allows an understanding to be obtained of the dynamic functionality of the said gene or genes in the cells.

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METHOD FOR DETECTING THE EXPRESSION OF BIOREGULATORY GENES IN MICROCULTURES OF EUKARYOTIC CELLS AND KIT THEREFOR

FIELD OF THE INVENTION

The present invention relates to a method for detecting the expression of bioregulatory genes in microcultures of eukaryotic cells. The present invention is particularly useful in diagnosis as well as gaining basic understanding of genesis and dynamics of viral, bacterial, fungal, oncogenic or immunoregulatory diseases. It is also useful for monitoring the effectiveness of therapies therefor. Diagnostic kits for carrying out the invented method are also provided.

Cytokines, soluble cell products that behave as local hormones, regulating the growth and behaviour of other cells, are involved in proper physiological functioning. Cytokines include the interferons, interleukins, tumor necrosis factors and similar substances, and the growth factors.

Study of cytokines has led to an important medical development of biotherapy. Cytokine biotherapy offers improvement over the existing treatment of cancer, viral, fungal, bacterial and immuno-regulatory diseases.

Eukaryotic cells such as lymphocytes, monocytes, fibroblasts, keratinocytes, astrocytes, endothelial cells, glial cells and platelets, are producing cytokines in response to various biological needs and functions.

In eukaryotic cells, gene expression involves the formation of messenger RNA during the process of protein synthesis. Therefore, it is possible to measure gene expression by quantitatively determining the extent of presence of mRNA specific for various cytokines.

Currently, there exists no direct method of assessing the dynamics of the immune response in a given patient, even though this information could be of great clinical

importance in diagnosis and the guiding of therapy in many diseases, including cancer, autoimmune diseases and immunodeficiency syndromes. Monoclonal antibodies can be used to count subsets of T cells, but fail to report the function and dynamic responsiveness of the immune system. Three human genes are of key importance in determining the strength of the immune response. They are encoding interleukin-2 (IL-2) or T-cell growth factor, the receptor for IL-2 (IL-2R), and immune-interferon or gamma interferon (IFN-y). The expression of these genes, induced by an immune stimulus, is normally down-regulated severely (up to 99%) by novel molecular mechanisms [Efrat, S. and Kaempfer, R. Proc. Natl. Acad. Sci. USA 81, 2601-2605 (1984); Efrat, S., Zelig, S., Yagen, B. and Kaempfer, R. Biochem. Biophys Res. Commun. 123, 842-848 (1984); Kaempfer, R. and Efrat, S. Cellular and Molecular Biology of Lymphokines (C. Sorg and A. Schimpl, eds.) Academic Press, Orlando, Fla., pp. 605-618 (1985); Kaempfer, R., Efrat, S. and Marsh, S. Molecular Cloning and Analysis of Lymphokines (D.R. Webb and D. Goeddel, ets.) Academic Press, Orlando, Fla., pp. 59-72 (1987); Lebendiker, M.A., Tal, C., Sayar, D., Pilo, S. Eilon, A., Banai, Y. and Kaempfer, R. EMBO J. 6, 585-589 (1987)] elicited, in part, by interactions between subsets of cells in the immune system [Kaempfer, R., and Efrat, S. Leukocytes and Host Defense (J.J. Oppenheim and D.M. Jacobs, eds.) Alan R. Liss, Inc., NY, pp. 57-68 (1986); Lebendiker, M.A., Tal, C., Sayar, D., Pilo, S., Eilon, A., Banai, Y. and Kaempfer, R. EMBO J. 6, 585-589 (1987); Kaempfer, R., Sayar, D., Efrat, S., Lebendiker, M., Ketzinel, M. and Tal, C. Lymphokine Research 6, Vol. 1 5th International Lymphokine Workshop Abstr. 1640 (1987)]. Moreover, expression of these three genes is functionally linked, creating a network of interacting genes that are expressed in different lymphocytes.

Conventional methods for the recovery of RNA from cells generally include lysis of the cells and ultracentrifugation through a cesium chloride gradient, are time consuming, cumbersome and require large numbers of cells and heavy equipment. Procedures known to be suitable for small numbers of cells, when applied to peripheral blood cells, suffer from the deficiencies that the quantitative determination of the RNA encoded by a specific gene is not sufficiently accurate to allow precise measure to be made of gene expression.

Conventional SDS and NP-40 methods of extraction of mRNA [White, B.A., S. Bancroft F.C. J. Biol. Chem. 257:8569 (1982)] are inappropriate for the detection of cytokines because the presence of contaminating protein masks the presence of RNA and some degradation of RNA occurs during the many steps involved in these procedures.

Guanidinium thiocyanate (GCN) Direct Blot [Matsuyama M, Sugamura K, Kawade Y. and Hinuma Y.J. Immunology 129:450 (1982)] is inappropriate because the viscosity of DNA interferes with the processing of RNA fractions. This procedure has the limitation that no more than 10^5 cells can be analyzed, too few for the detection of cytokine gene expression which is at lower levels than that of most other genes.

Other methods of recovering RNA based on guanidinium, such as GCN-LiCl (Cathala G. Savouret J.F. Mendez B. West B.L., Karin M., Martial J.A. and Baxter J.D. DNA 2:239 1983) are yielding a pure mRNA but involve too many steps and cause some loss of RNA to allow convenient processing of large numbers of samples.

A method based on guanidinium hydrochloride [Cheley S. and Anderson R. Anal. Biochem. 137:15 (1984)] has been used to detect viral RNA expressed in situations of chronic viral infections. The present inventors have discovered that an adaption of this method can be used for

detecting the expression of bioregulatory genes in eukaryotic cells provided that appropriate steps are taken to induce expression of the bioregulatory gene or genes. The method according to this invention can be used in order to identify and quantify reliably and repeatedly the expression of bioregulatory genes in only a few millilitres of peripheral blood taken from a given patient. Further, the present inventors have discovered that by using a plurality of induction processes on separate samples of the eukaryotic cells a much greater understanding can be obtained of the dynamic functionality of the genes in the cell sample than is possible by any known technique.

Therefore, the present inventors have selected and provided a method which overcomes the shortcomings of the prior art techniques for measuring gene expression in cytokine-producing cells, inducing the expression of cytokine cells and detecting such expression in a smaller number of cells than hitherto possible, in defined culture conditions that are capable of providing measurement not only of actual expression of cytokine genes but also of the functional intactness of regulatory mechanisms that control expression of these genes and their disturbance in pathological situations. The object of the invention is to provide a highly efficient method of mRNA extraction from appropriately cultured human peripheral blood mononuclear cell populations which increases the sensitivity for detecting cytokine gene expression and avoids the pool artifacts created by the use of standard Northern blot analyses, in order to assess the normal or abnormal expression of cytokine gene and their regulation.

SUMMARY OF THE INVENTION

The present inventors have now succeeded in providing a method that allows accurate and sensitive detection of a transient expression of bioregulatory genes in situations

where such expression is usually very low and almost undetectable and where only small cell samples are available.

The technique according to this invention can, in preferred embodiments, accurately monitor the extent of gene expression in as little as half-a-million cells or up to 500-fold fewer cells than needed for the standard methods that have hitherto been used to detect the expression of such genes. This allows the study of the expression of various bioregulatory genes in only a few millilitres of peripheral blood which are easy to get from a patient and which express many genes of key bioregulatory importance.

It is an object of the present invention to provide a general method for detecting the expression of bioregulatory genes in microcultures of eukaryotic cells, having an important diagnostic and therapeutic value comprising the steps of:

(a) isolating and culturing said cells under conditions inducing the expression of at least one of said genes;

(b) lysing said cultured cells with guanidinium hydrochloride;

(c) selectively precipitating ribonucleic acid by adding to the homogenized cell preparation a sodium or potassium acetate solution followed by absolute ethanol;

(d) recovering and blotting RNA onto support matrix;

(e) hybridizing recovered and blotted RNA with a labelled riboprobe specific to at least one of said genes; and

(f) quantitatively determining the extent of binding of said labelled riboprobe to said blotted RNA.

In another preferred embodiment, said lysing step (b) is done by adding guanidinium thiocyanate and said selective precipitation in step (c) by addition of lithium

chloride followed by sodium or potassium acetate solution and absolute ethanol.

The method according to the present invention is preferably carried out for the detection of expression of bioregulatory genes, selected from the group comprising interferons, interleukins, tumor necrosis factors, and growth factors, wherein the eukaryotic cells comprise lymphocytes, monocytes, keratinocytes, fibroblasts, astrocytes, endothelial cells, glial cells and platelets. Inducing the expression of the bioregulatory genes is preferably carried out by a compound such as phytohemagglutinin or concavalin A, with or without cycloheximide and gamma-irradiation, or their appropriate combination. Gamma-irradiation is an example of a treatment that prevents the activation of suppressor T cells [Gullberg M., Larsson E.L., J. Immunol. 128:746 (1982)], which affects IFN-y gene expression [Lebendiker, M.A., Tal C., Sayer D., Pilo S., Banai Y., Eilon A. & Kaempfer R. EMBO 6:658 (1987)]. Cycloheximide is an agent that leads to abnormal expression of the IL-2 gene [Efrat S. and Kaempfer R. Proc. Natl. Acad. Sci. USA. 81; 2601 (1984)] and the IFN-y gene [Lebendiker, M.A., Tal C., Sayar D., Pilo S., Banai Y., Eilon A. & Kaempfer R. EMBO 6:585 (1987)].

The process according to the present invention is preferably carried out on two separate aliquots of a single sample. One aliquot is probed without inducer having been added to give a reading representing a basal expression level while the second aliquot is subjected to inducement by a suitable inducer such as phytohemagglutinin. The ratio of the induced expression level to the basal level indicates whether gene regulation is normal or not. If desired inducement may be carried out on separate aliquots of the cell sample to provide kinetic information on the induction process.

In a further preferment according to this invention additional or alternative inducers such as cycloheximide are added to additional aliquots of the cell sample to super induce the cells. Such superinduction can indicate the maximum potential for gene expression. This in turn provides a guide as to the extent to which the basal expression level approaches the maximum potential expression of the gene.

It has been found that suppressor T cells down-regulate at least some cytokine genes. Y-irradiation prevents activation of suppressor T cells. Thus, advantageously, one aliquot of the cell-sample is subjected to superinduction and Y-irradiation. Such a sample will indicate the extent to which suppressor T cells are down-regulating expressions of the gene of interest.

The method according to this invention requires the said riboprobe to be labelled with a detectable isotopic or non-isotopic chemical group.

The invention offers convenient analysis of many samples (48 and more) without the use of heavy equipment and a rapid quantitation of data in numerical form.

The method according to this invention may be carried out conveniently and without requiring the use of an ultracentifuge, making the rapid analysis of large numbers of samples feasible; moreover, it reproduceably detects gene expression over at least a 200-fold range. The equipment needed is standard and simple in nature: microfuge, cell centrifuge, CO_2 incubator, dot-blot apparatus, waterbath.

A 10-ml sample of blood yields usually 10^7 cells, permitting multiple analysis of gene expression (several genes, several conditions of induction). The ability to make many measurements on a single blood sample allows refinements under various conditions of induction to

measure intactness of gene regulatory mechanisms in disease.

Response is linear with increasing RNA input. Response increases linearly both with cell number and amount of RNA. Three times more cells generate a signal equal to that of three times more RNA, i.e., threefold stronger. The gene expression signal is linear over at least a 200-fold range. Thus, both hybridization and film blackening are a quantitative measurement. These properties render the method highly reproducible and informative.

The product is total cell RNA and mRNA intact with no more than 5% contaminating DNA and even less protein.

There is no masking of the signal of hybridization by protein. The hybridization signal is equally as strong as that of pure RNA prepared by guanidinium thiocyanate lysis and ultracentrifugation through CsCl, a long and cumbersome procedure.

A signal of specific mRNA, e.g., for Interleukin-2 (IL-2) or Interferon-gamma (IFN-y), can be detected in as little as 5 x 10^5 cells within 24hr of film exposure. This is 20-to-100 fold fewer cells than are needed for a standard Northern blot signal for these genes.

DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagrammatic outline of a procedure according to this invention;

Fig. 2 demonstrates the use of the assay for measuring IFN-y gene expression. 4 x 10^6 human peripheral blood mononuclear cells were induced for IFN-y gene expression. RNA extracted from the cells was analyzed in four serial dilutions (0.125, 0.25, 0.5 and 1) using a manifold dot-blot device. The nitrocellulose filter was hybridized to IFN-y riboprobe. Gamma-irradiation (Y-irradiation) was done before addition of 0.4% (v/v) phytohemagglutinin (PHA), the dose was 1500

rad. Cycloheximide (CHX) was added to several cultures after the addition of PHA. The filter was exposed to film after hybridization and the autoradiogram is shown.

Fig. 3 is a graph showing the linearity of the assay according to this invention. Samples expressing different levels of IL-2 RNA or IFN-y RNA were analyzed in four serial dilutions (0.125, 0.25, 0.5 and 1.0) using a manifold dot-blot device. After hybridization to specific riboprobes and autoradiography of the nitrocellulose filters, absorbency of individual dots at 630nm was measured in a micro-elisa autoreader.

Fig. 4 is a graph showing the linearity and equivalent response of the assay as a function of increasing cell number or increasing RNA input. 4 x 10^6 cells, induced for IL-2 gene expression, were serially diluted and RNA was then extracted (▲). Alternatively, RNA was extracted from 4 x 10^6 cells, and then serially diluted (o).

Fig. 5 is a graph showing the kinetics of IL-2 mRNA levels in human peripheral blood lymphocytes (PBL) induced with phytohemagglutinin (PHA). Cells were induced with PHA (0.4% v/v) at 0hr. Samples of 4 x 10^6 cells were taken at each time point indicated, and RNA was extracted. Cycloheximide (CHX; 20ug/ml) was added at 21hr and RNA was analyzed at 24hr (▲).

Fig. 6 is a chart showing the quantitation of IL-2 and IFN-y gene expression in PBL of nine blood donors. 10ml of peripheral blood was taken from each donor. Cells from each donor were incubated with 1) no additions (background, BKG); 2) addition of 0.4% (v/v) PHA; 3) addition of PHA after Y-irradiation (1500 rad) of the cells; 4) addition of PHA, followed, at 16hr, by the addition of CHX (20ug/ml). RNA was extracted from all samples of 20hr after the addition of PHA and dot-blot filters were hybridized separately to IL-2 and IFN-y

riboprobes. Bars denote film density for respective assays, determining in a micro-elisa autoreader.

Fig. 7 is a chart showing the distribution of IL-2 and IFN-y gene expression levels in cells from the nine donors described in Fig. 6, expressed as absorbency of film at 630mm which reflects the amount of hybridized RNA.

A preferred method according to the present invention is as follows:

Example 1 - Culture of Lymphocytes

1. Take 10ml of peripheral blood from patient in heparized tube.

2. Dilute 2-fold with phosphate-buffered saline (PBS).

3. Layer over 2vol Ficoll (Pharmacia).

4. Spin 30 min at 200g at room temperature in cell centrifuge.

5. Wash twice with RPM1 1640 medium without serum.

6. Resuspend cells at a density of 4×10^6 in 10-ml screw cap round-bottom plastic culture tubes (Cel-Cult) into RPM1 1640 medium containing:

2 mM glutamine
10 mM MEM non-essential amino acids
100 mM MEM sodium pyruvate
10 mM HEPES pH 7.2
100 u/ml penicillin
100 ug/ml streptomycin
40 ug/ml gentamycin
5×10^{-5} M 2-mercaptoethanol
2% fetal calf serum
5 ug/ml nystatin

7. Incubate overnight at 37°C in an atmosphere of 5% CO_2.

Example 2 - Induction Conditions

Cells from each donor were incubated with 1) no additions (background, BKG): 2) addition of 0.4% (v/v)

PHA; 3) addition of PHA after Y-irradiation (1500 rad) of the cells; 4) addition of PHA, followed, at 16hr, by the addition of CHX (20ug/ml). RNA was extracted from all samples at 20hr after the addition of PHA.

Example 3 - Analytical Procedure. RNA Extraction

All steps are performed in a single microcentrifuge tube of 1.5ml, using a standard table-top microcentrifuge (Eppendorf).

1. Spin down 1ml of cell suspension in an Eppendorf centrifuge at 500rpm for 5 min.

2. Add 0.4ml of 7.5M guanidinium thiocyanate (GTC) may be used as described in Example 4.

3. Homogenize by vortexing the tube for at least 30 sec, until dissolved completely.

4. Add 20ul of 2 M KOAc pH 5.1. Mix and then add 250ul of absolute ethanol. Mix again.

5. Leave overnight at -20°C.

6. Centrifuge at 15,000 rpm for 30 min at 4°C in an Eppendorf centrifuge.

7. Discard supernatant.

8. Dissolve pellet into 50ul of 37% formaldehyde. Mix on a vortex until dissolved. Then add 50ul of 3 M NaCL, 0.3 M Na-citrate.

9. Place for 15 min at 60°C.

10. Perform a dot-block procedure. The sample is pipetted into a single well of a 96-place microtiter plate (Nunc) and serially diluted, in 2-fold steps, into 1.5 M NaCl, 0.15 M Na-citrate (10 x SSC) and pipetted into adjacent wells. After all samples have been diluted, the contents of each well of a 96-place manifold dot-blot device (Schleicher & Schuell) under vacuum, containing a 9 x 13cm nitrocellulose sheet (Schleicher & Schuell GB 003 blotting paper. The nitrocellulose sheet and the blotting paper were presoaked in distilled water and then in 10 x SSC. Each well is washed once with 100ul of 10 x SSC.

The nitrocellulose is baked at 80°C for 2hr under vacuum and probed with the desired gene riboprobe, as described in examples 5 or 6.

11. Quantitate film dots in micro-elisa autoreader. To this end, exposed and developed X-ray film is cut into the shape of a microtiter plate, placed on the plate such that dots align with wells, and introduced into the autoreader (e.g., Dynatech). Scanning is at a wavelength of 630nm.

Example 4 - Alternative Procedure of RNA Extraction

1. Spin down 1ml of cell culture in an Eppendorf centrifuge at 500rpm for 5 min.

2. Discard supernatant and drain pellet.

3. Add 100ul of lysis buffer, consisting of 5 M GTC, 10 mM EDTA, 50 mM Tris-HCl pH 7.5, and 8% (v/v) 2-mercaptoethanol (added freshly).

4. Mix three times on vortex for 10 sec. Add 700ul of 4 M LiCl and leave for 20hr (overnight) at 4°C.

5. Spin in an Eppendorf centrifuge for 90 min at 12,000 rpm at 4°C.

6. Discard supernatant, dry pellet and 50ul of RNA solubilizing buffer: 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 10 mM Tris-HCl pH 7.5

7. Mix by vortexing for 20 sec. Repeat after every 10 min for 40 min. This step is done at room temperature.

8. Extract solution by vortexing it with an equal volume of phenol/chloroform (1:1), collect upper phase.

9. Add 1/10 vol of 3 M NaOAc pH 5.1, mix, add 2.5 vol absolute ethanol and leave at -20°C for 20 hr.

10. Precipitate RNA at 4°C by a 45 min centrifugation at 15,000 rpm in an Eppendorf centrifuge.

11. Wash pellet with 70% ethanol.

12. Discard supernatant and dry pellet.

13. Follow steps 8-11 of Example 3.

Example 5 - Preparation of Riboprobe of IL-2

The riboprobe is synthesized using Promega-Biotec riboprobe kit. IL-2 cDNA was excised from plasmid p3-16 (Taniguchi T., Matsui, H., Fujita, T., Takaoka, C., Kashima, N., Yoshimoto, R., Hamuro, J. Nature 302 (1983) p.305) with Pst I and inserted into pGEM-3 (Promega Biotec) at the Pst I site. The orientation in which transcription from the T7 promoter gives the desired RNA strand was selected.

a) Transcript was made as follows:-

1) 4ul transcript buffer.

2) 2ul dithiotreitol (DDT) (100 mM).

3) 0.6ul RNasin (350 u/ml).

4) 1ul each of the nucleotides UTP, CTP, GTP (0.5 mM).

5) 1ul T7 RNA polymerase (15 u/ml).

6) 1ul plasmid containing the IL-2 cDNA (1 ug/1 ul.

7) 10ul [α-^{32}P] ATP (10 mCi/ml).

8) 10ul of [α-^{35}] ATP (10 mCi/ml) (Amersham) may be substituted for [α-^{32}P] ATP in the previous step.

b) The mixture is incubated for 1hr at 37°C.

c) 1ul DNase (free of RNase; 1u/ml is added and incubation is continued for 15 min at 37°C.

d) RNA is extracted as follows:

1) 20ul of phenolchloroform (1:1) is added, followed by mixing on vortex and centrifugation for 2 min in an Eppendorf centrifuge.

2) The upper (aqueous) phase is carefully collected.

3) The lower phase of phenochloroform is washed with 20ul of TE buffer: 10 mM Tris-HCl pH 7.6, 1 mM EDTA.

4) After vortexing and centrifugation of 2 min in an Eppendorf centrifuge, the upper phase is collected and added to the aqueous phase collected previously.

5) 40ul of chloroform is added to the combined aqueous phases.

6) After vortexing and centrifugation for 2 min in an Eppendorf centrifuge, the upper phase is collected.

7) The lower phase is extracted with 40ul of TE buffer, as described under steps 3 and 4 above, and the aqueous phase is combined with the aqueous phase collected in step 6 above.

8) 8ul of 3 M NaOAc pH 5.1 and 2.5 vol of absolute ethanol are added to the combined aqueous phases.

9) The solution is stored for 20 hr (overnight) at -20°C, or for 2 hr at -70°C.

10) Centrifuge for 30 min at 4°C in an Eppendorf centrifuge at 15,000 rpm.

11) Wash pellet with 1ml of 70% ethanol.

12) Dry pellet in vacuo.

13) Resuspend pellet in 100ul TEN buffer containing: 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.1 M Na Cl and 20 ug/ml tRNA from E.coli MRE 600 (Boehringer).

14) Apply to a 1-ml Sephadex G-50 column made in a 2-ml syringe and equilibrated with TEN buffer.

15) Centrifuge the column for 5 min at 1,000 rpm in a cell centrifuge and collect the flow-through in a tube.

16) Take a 1ul sample into 10% ice-cold trichloroacetic acid and determine precipitable radioactivity.

17) A good preparation yields 3 - 10 x 10^5 cpm/ul.

Example 6 - Preparation of Riboprobe for IFN-γ

The riboprobe for IFN-γ gene expression was synthesized following the procedure described in Example 5. IFN-γ cDNA was excised from plasmid pH11F-SV-γ with Bam H1 (Devos, R., Cheroutre, H., Taya, Y., Degrave, W., Heuverswyn, H.Y., Fiers, W. Nucl. Acids Res. 10 (1982) p.2487) and inserted into pGEM-3 at the Bam H1 site. The orientation in which transcription from the T7 promotor gives the desired RNA strand was selected.

Example 7 - Hybridization of Nitrocellulose Filter

1) Filters are prehybridized in a sealed plastic bag for 4 hr at 60°C. The prehybridization buffer contains:
25% formamide
10% dextran sulfate (5,000 mol weight)
1% SDS
50 mM Tris-HCl pH 7.5
100 ug/ml salmon sperm DNA that has been boiled for 10 min and cooled rapidly
100 ug/ml tRNA (E.coli MRE 600)
0.7 M NaCl

2) For hybridization add:
5 x 10^6 cpm/ml of riboprobe (see above) x 1
Denhardt solution: 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinyl-pyrrolidone, 0.02% (w/v) BSA fraction V (Sigma)
100 ug/ml tRNA (E.coli MRE600)
Incubate for 16-18 hr, but not more, at 60°C.

3) After hybridization, filters are washed:
1) twice with 2 x SSC (500 ml) at room temperature for 15 min
2) twice with 2 x SSC, 1% SDS (500 ml) at 68°C for 30 min
3) once with 0.2 x SSC, 0.2% SDS (500 ml) at 68°C for 30 min.

Filters are dried and exposed to X-ray film for 5-25 hr at -70°C.

ANALYSIS OF RESULTS

Results may be analyzed as follows:

	IL-2	IFN-Y
No inducer	1A	1B
PHA (16 hr)	2A	2B
PHA (20 hr)	3A	3B
PHA + cycloheximide	4A	4B
PHA + Y-irradiation	5A	5B

(a) Basal level of gene expression:

1A, 1B.

(b) Inducibility of genes

Ratio 2A/1A, 2B/1B. Ratio 3A/1A, 3B/1B.

The difference between samples 2 and 3 is kinetic: transient expression of these genes may peak by 16 hr or 20 hr, depending on the donor. Normally, these values should be 3-10; if less, this means either that the genes are highly activated, or that they fail to respond to induction. These possibilities can be distinguished in step (c) below.

(c) Superinducibility by cycloheximide:

A4/A3, B4/B3.

The ratio shows the potential for gene expression. Normally, this ratio should be considerably greater than 1 (1.5-5 or more). If not, this means that either basal expression is very high, in which case the ratios A4/A1, B4/B1 should be close to 1, or, alternatively, that induced gene expression is very high and uncontrolled, in which case the ratios A3/A1 and B3/B1 should be high.

(d) Superinducibility by Y-irradiation:

A5/A3, B5/B3.

Normally, this ratio should be well in excess of 1 (1.5-3). Normally, the ratio B5/B3 will exceed the ratio A5/A3. These values measure the extent to which suppressor T cells down-regulate IL-2 and IFN-Y gene expression; the latter gene is more strongly regulated in this manner. The higher these ratios, the stronger the down-regulation.

CLAIMS:-

1. A method for detecting the expression of bioregulatory genes in microcultures of eukaryotic cells, comprising the steps of:

(a) isolating and culturing said cells under the conditions inducing the expression of at least one of said genes;

(b) lysing said cultured cells with guanidinium hydrochloride;

(c) selectively precipitating ribonucleic acid by adding to the homogenized cell preparation sodium or potassium acetate solution followed by absolute ethanol;

(d) recovering and blotting RNA onto support matrix;

(e) hybridizing recovered and blotted RNA with a labelled riboprobe specific to at least one of said genes; and

(f) quantitatively determining the extent of binding of said labelled riboprobe to said blotted RNA.

2. The method according to Claim 1, wherein said lysing step (b) is done by adding guanidinium thiocyanate and said selective precipitation in step (c) by addition of lithium chloride followed by sodium or potassium acetate solution and absolute ethanol.

3. The method of Claims 1 or 2, wherein the bioregulatory gene is selected from the group comprising those genes coding for the interferons, interleukins, tumor necrosis factors, and growth factors.

4. The method of Claim 3, wherein the eukaryotic cells are selected from the group comprising lymphocytes, monocytes, fibroblasts, astrocytes, endothelial cells, glial cells, keratinocytes and platelets.

5. The method of Claim 4, wherein the inducer of said expression is a compound selected from a group comprising phytohemagglutinin, concavalin A, cycloheximide, or their appropriate combination with or without Y-irradiation.

6. The method of Claim 5, wherein said riboprobe is labelled with a detectable isotopic or non-isotopic chemical group.

7. A method for detecting the presence of RNA translatable into at least one cytokine, comprising the steps of:

(a) providing at least 10 milliliters of peripheral blood;

(b) isolating and culturing at least 4 cultures of about 1 x 10^6 lymphocyte cells;

(c) inducing said cells to produce at least one cytokine;

(d) lysing said induced cells with 7.5 M guanidinium hydochloride;

(e) precipitating RNA from the homogenized cell lysate at -20°C by adding salts to final concentration of 0.1 molar solution of sodium or potassium acetate followed by absolute ethanol;

(f) centrifuging and recovering RNA in essentially purified form;

(g) blotting RNA onto support matrix; and

(h) hybridizing RNA to a labelled riboprobe specific to at least one cytokine;

(i) measuring in a micro-elisa autoreader the absorbency of film blackened by individual dots of the nitrocellulose filters to determine the presence and quantitate the amount of said RNA.

8. The method according to Claim 7, wherein lysing of said induced cells in step (d) is done with 5M guanidinium thiocyanate and precipitation of said RNA in step (e) by addition of 3.5 M lithium chloride followed by 0.3 M of sodium or potassium acetate followed by absolute ethanol.

9. The method of Claim 7 or 8, wherein said cytokine is a polypeptide selected from the group comprising IL-2 and IFN-y.

10. The method of Claim 9, wherein in the step (c) said cells are incubated with 0.4% (v/v) phytohemagglutinin for about 16 hours, at which time 20 ug/ml of cycloheximide is added and incubation is continued for another 4 hours.

11. The method of Claim 9, wherein in the step (c) said cells are incubated with 0.4% (v/v) PHA alone or following 1500 rad of Y-irradiation of said cells to induce the production of at least one cytokine.

12. The method of Claim 7 or 8, wherein said riboprobe is labelled with a detectable isotopic or non-isotopic chemical group.

13. The method of Claim 7 or 8, wherein the said cytokine detection is used to diagnose viral, bacterial, fungal, oncogenic or immunoregulatory diseases.

14. A diagnostic kit for carrying out the method of any one of the Claims 1 to 13.

15. A disease monitoring kit for carrying out the method of any one of the Claims 1 to 13.

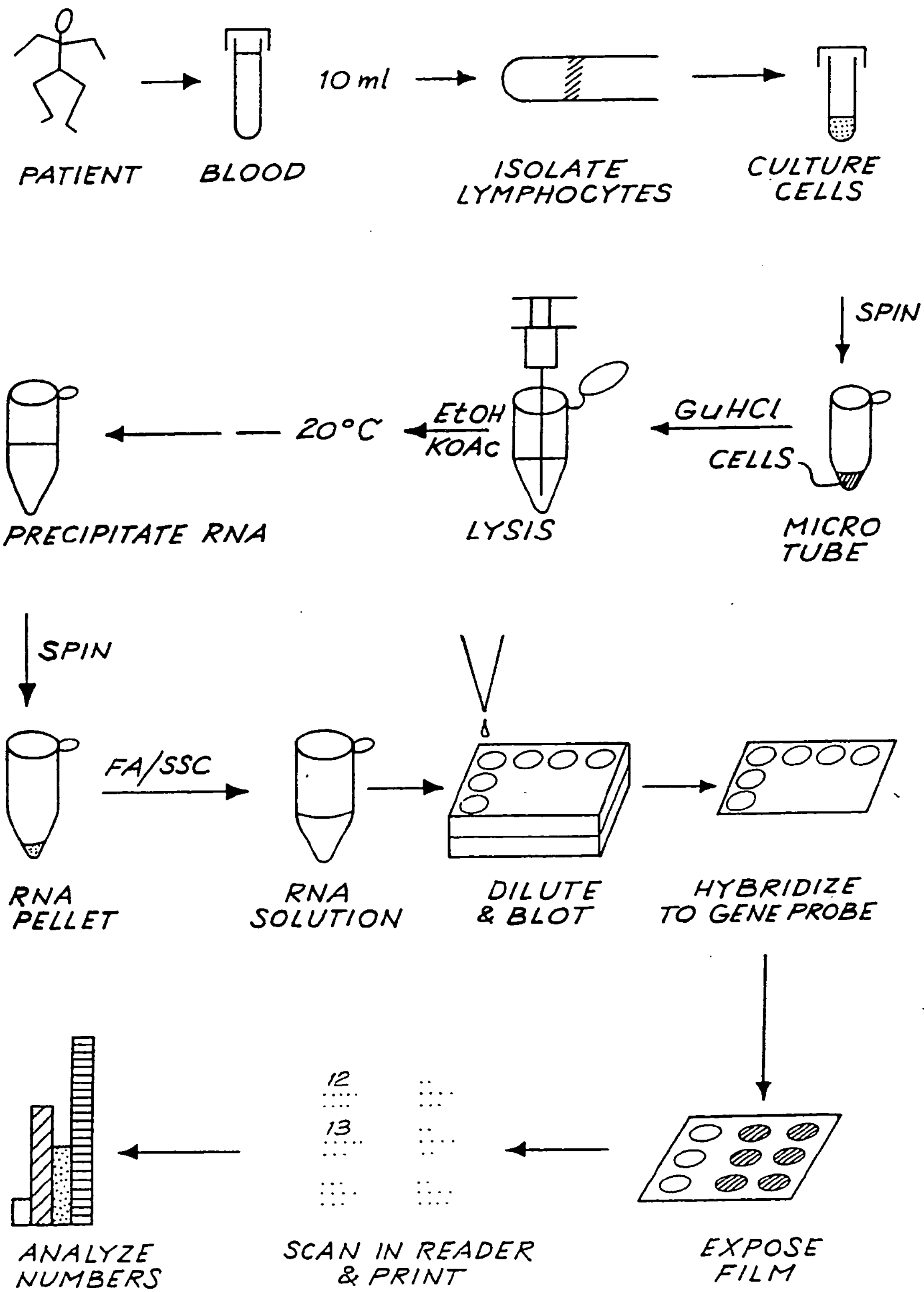
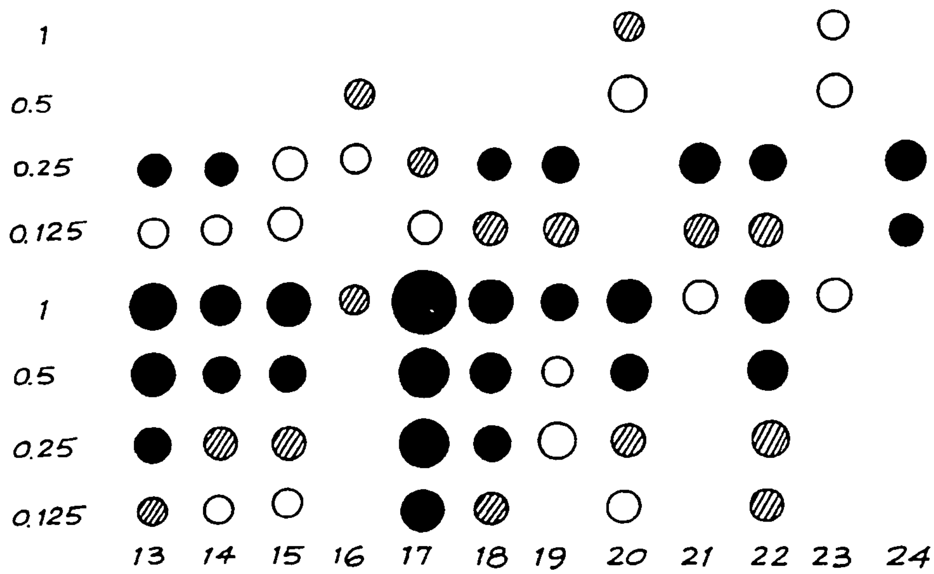


FIG. 1

SUBSTITUTE SHEET



FILM:	1	2	3	4	5	6	7	8	9	10	11	12
PHA	+	+	+	+	+	+	+	+	+	+	+	+
CHX (hr added) (20μg/ml)	-	1/2	-	-	1/2	1	-	-	1/2	1	2	-
γ-IRRADIATION	-	-	+	-	-	-	+	-	-	-	-	+
TIME of RNA EXTRACTION (hr)	1	1	1	2	2	2	2	4	4	4	4	4

	13	14	15	16	17	18	19	20	21	22	23	24
PHA	+	+	+	+	+	+	+	+	+	+	-	
CHX (hr added) (20μg/ml)	-	1/2	1	2	-	-	1/2	1	2	-	-	
γ-IRRADIATION	-	-	-	-	+	-	-	-	-	+	-	
TIME of RNA EXTRACTION (hr)	6	6	6	6	6	22	22	22	22	22	22	

SUBSTITUTE SHEET

FIG. 2

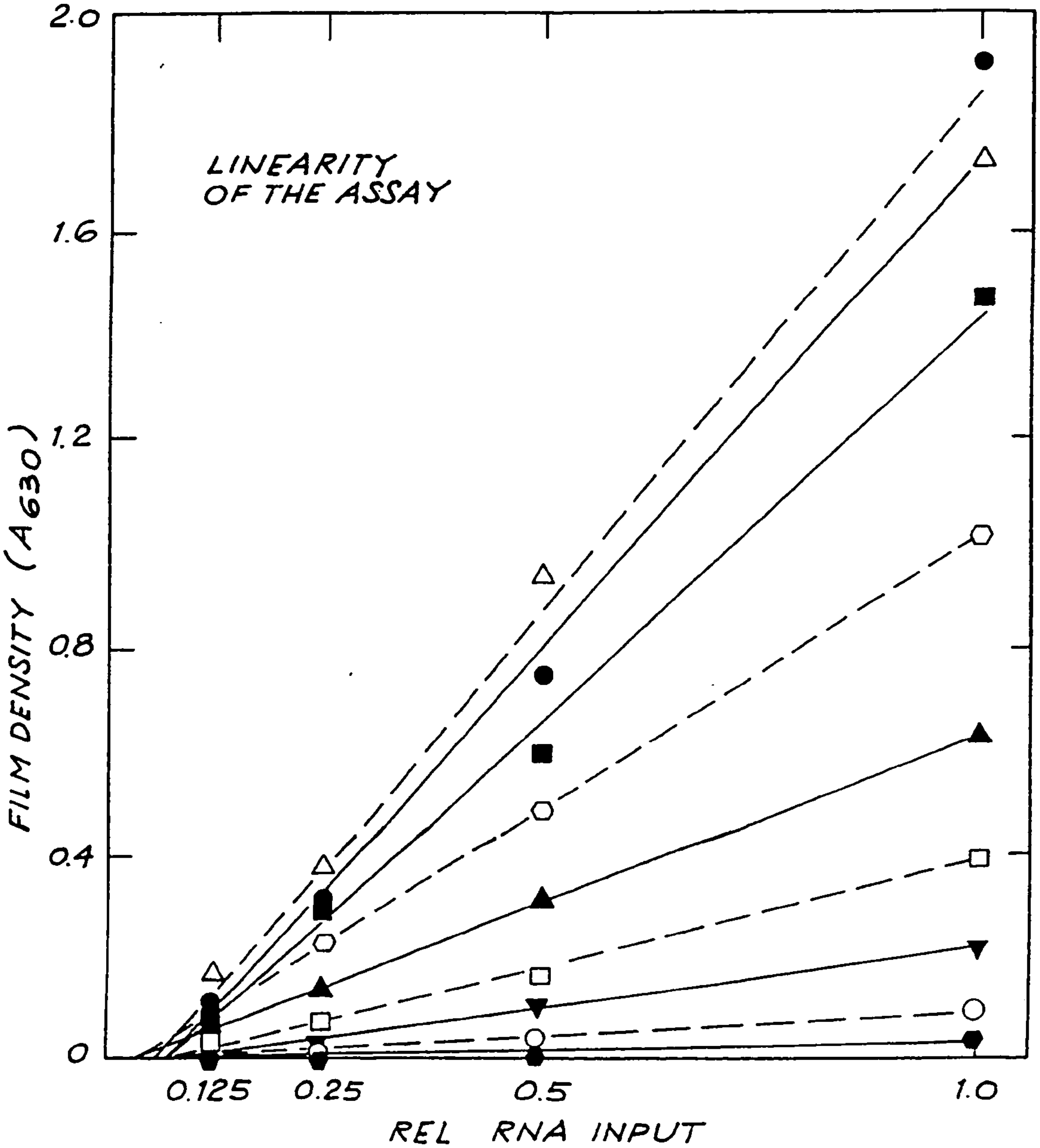

LINEARITY
OF THE ASSAY
2.0
1.6
1.2
0.8
0.4
0
FILM DENSITY (A_{630})
0.125
0.25
0.5
1.0
REL RNA INPUT

FIG. 3

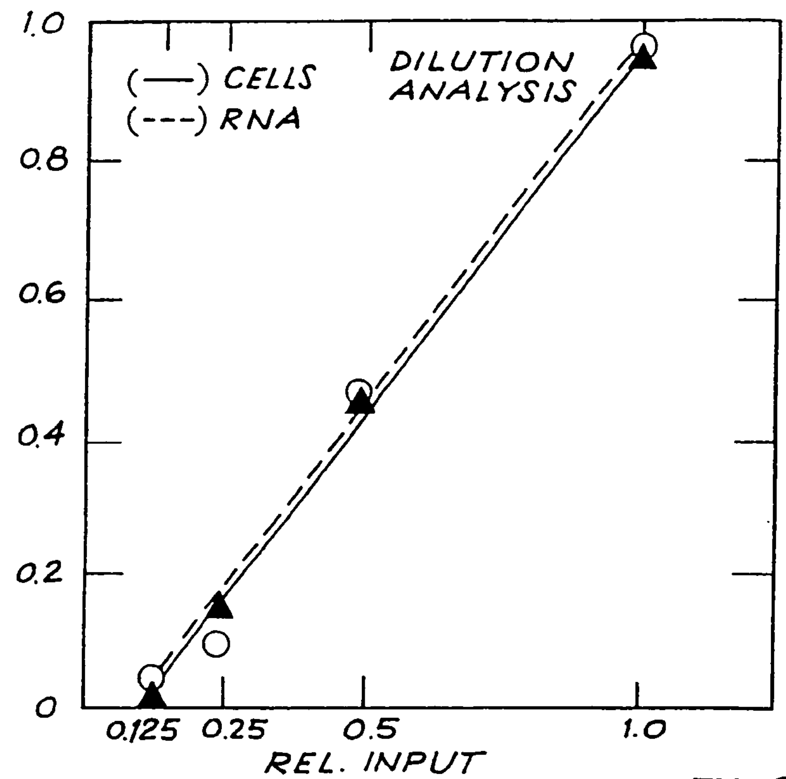

1.0
0.8
0.6
0.4
0.2
0
(—) CELLS
(---) RNA
DILUTION ANALYSIS
0.125
0.25
0.5
1.0
REL. INPUT

FIG. 4

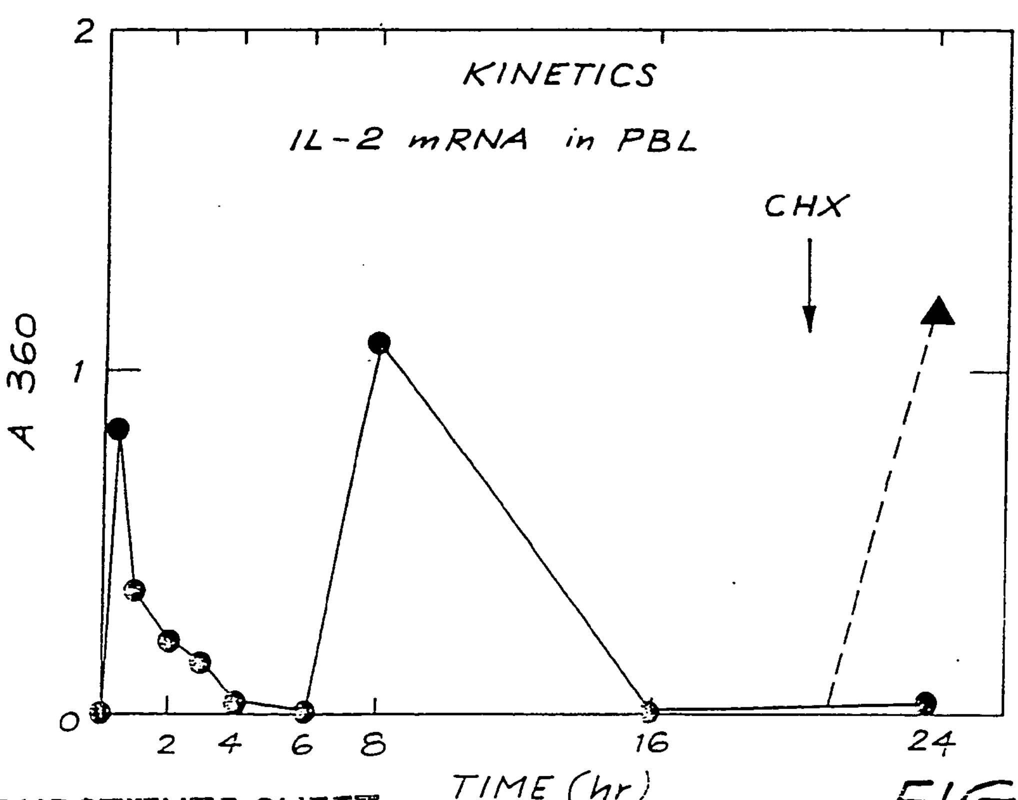

2
1
0
A 360
KINETICS
IL-2 mRNA in PBL
CHX
2
4
6
8
16
24
TIME (hr)

FIG. 5

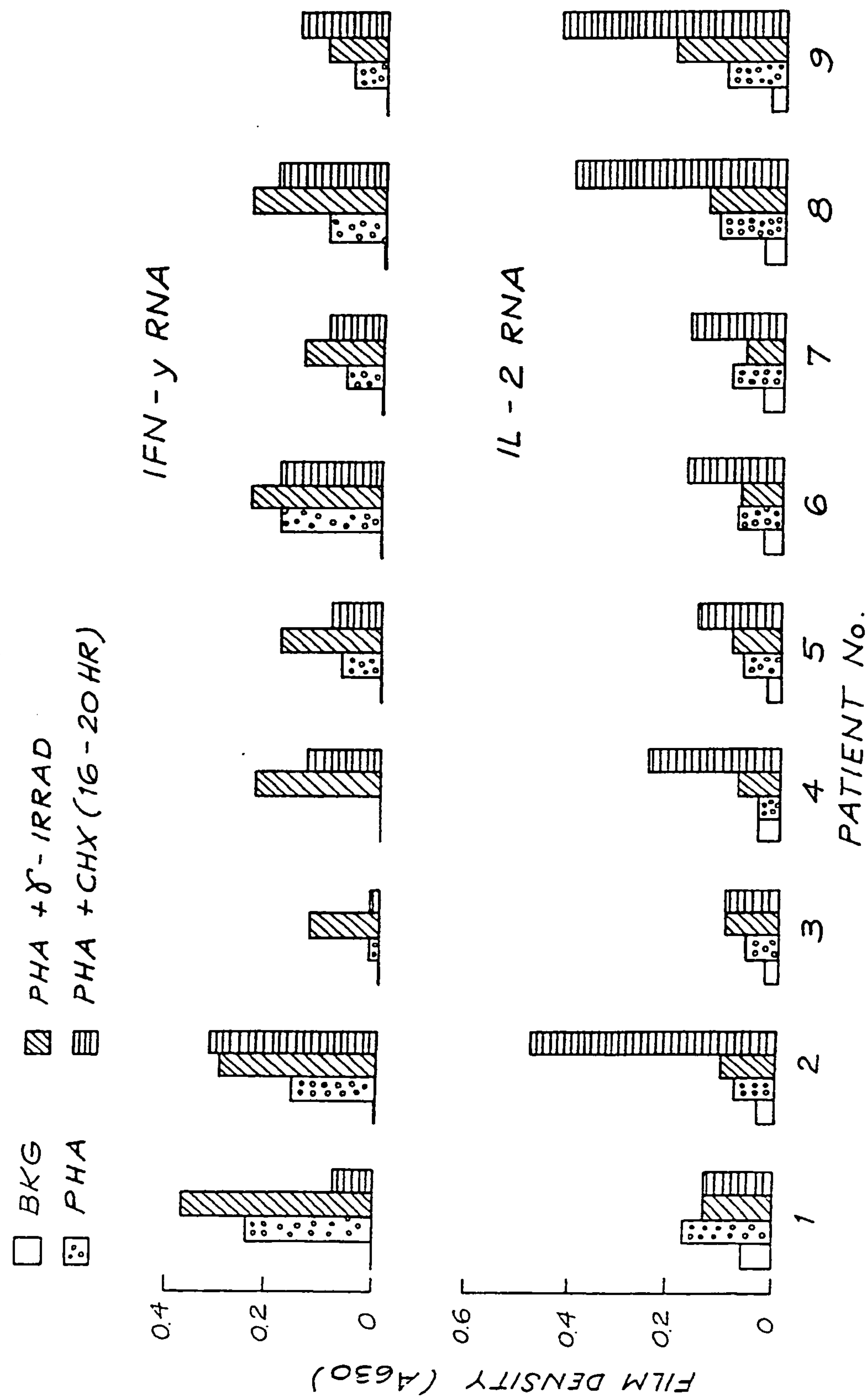

BKG
PHA
PHA + γ - IRRAD
PHA + CHX (16 - 20 HR)
IFN - γ RNA
IL - 2 RNA
FILM DENSITY (A630)
0.4
0.2
0
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
9
PATIENT No.

FIG. 6

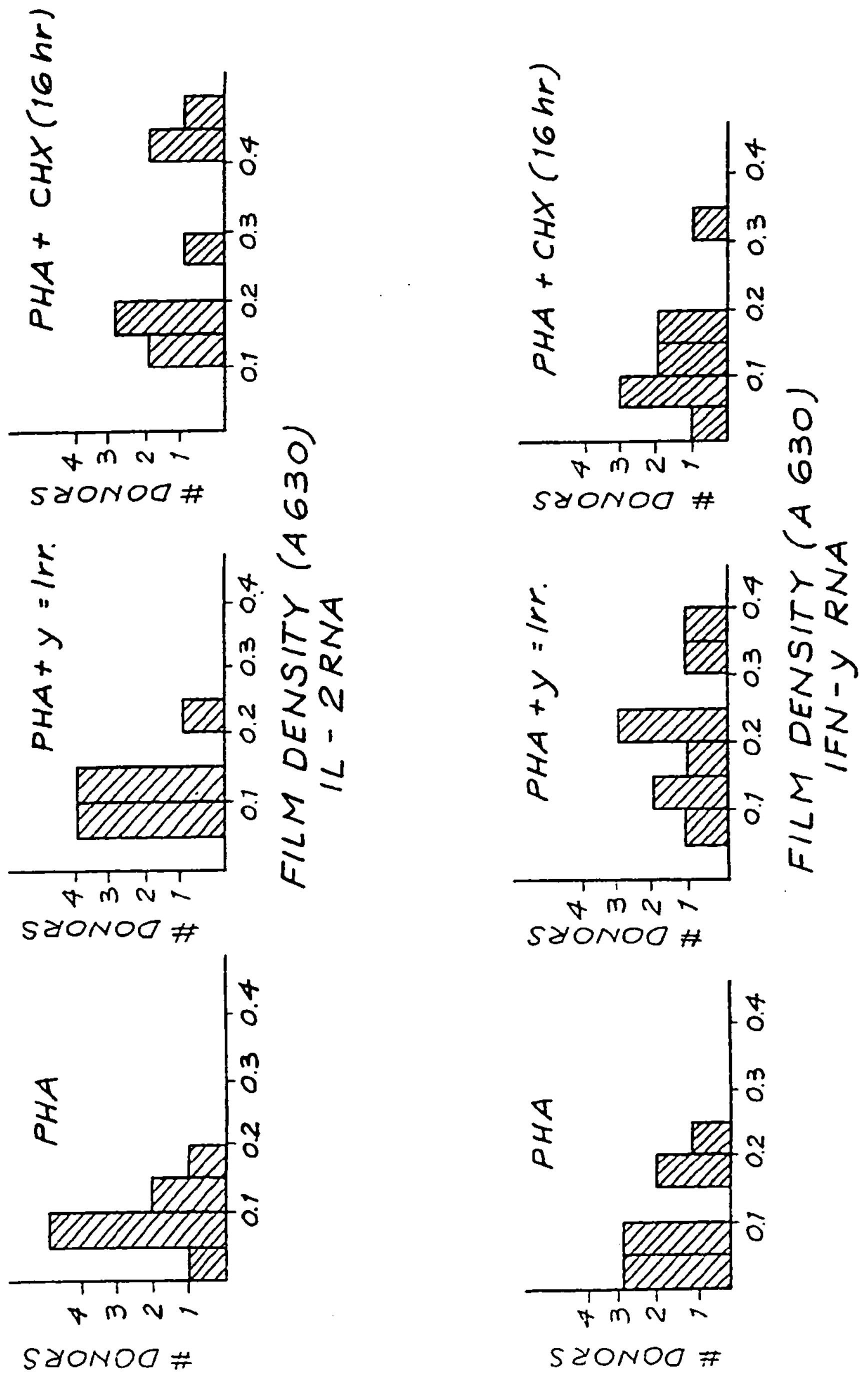

PHA
PHA + γ-Irr.
PHA + CHX (16 hr)
DONORS
4 3 2 1
0.1 0.2 0.3 0.4
FILM DENSITY (A630)
IL-2 RNA
PHA
PHA + γ-Irr.
PHA + CHX (16 hr)
DONORS
4 3 2 1
0.1 0.2 0.3 0.4
FILM DENSITY (A630)
IFN-γ RNA

FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 88/00099

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.^{4} C12Q 1/68, G01N 33/53

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^4	C12Q 1/68 USPA, WPI, WPIL) 'RNA' & 'PROBE' Chemical Abstracts)

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X,Y	Analytical Biochemistry, Volume 137 pages 15-19 (1984), S. Cheley and R. Anderson "A Reproducible Microanalytical Method for the Detection of Specific RNA Sequences by Dot-Blot Hybridization.	(1,13,15 & 2)
P,X	AU,A, 74329/87 (GILLESPIE, D.) 5 November 1987 (05.11.87)	(1-15)
X	US,A,4,483,920 (GILLESPIE, D., BRODSKY, I. BRESSER, J.) 20 November 1984 (20.11.84)	(1-15)
Y	The EMBO Journal, Vol.6 no.3 issued 1987 (IRL Press Ltd), Oxford, England, M.A. Lebendiker et al "Superinduction of the human gene encoding immune interferon" see pages 585-589.	(1-6)
Y	Proceedings of the National Academy of Sciences USA, Vol 81 issued 1984 May, S. Efrat and R. Kaempfer "Control of Biologically Active Interleukin 2 Messenger RNA Formation in Induced Human Lymphocytes" see pages 2601-2605.	(1-6)
	(continued)	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
5 July 1988 (05.07.88)	(28.07.88) 28 JULY 1988
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	JOHN ASHMAN Ashman

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	The Journal of Immunology, Vol.129 no.2 issued 1982 August (USA), M. Matsuyama et al "Production of Immune Interferon by Human Cytotoxic T Cell Clones" see pages 450-451.	(1,3-5)
Y	EP,A1, 0140764 (INSTITUT PASTEUR) 8 May 1985 (08.05.85) see abstract and claims.	(1-15)
Y	AU,A, 27546/84 (ENZO BIOCHEM INC.) 8 November 1984 (08.11.84) see examples IV, V and claims.	(1-15)

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers ...., because they relate to subject matter not required to be searched by this Authority, namely:

2.☐ Claim numbers ...., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.☐ Claim numbers.........., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/AU 88/00099

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
WO 8706621	AU 74329/87		
EP 140764	FR 2552879	JP 60102200	
AU 27546/84	DK 2178/84 ES 8600404 NO 841725	EP 124124 IL 71699	ES 532041 JP 60040956

END OF ANNEX

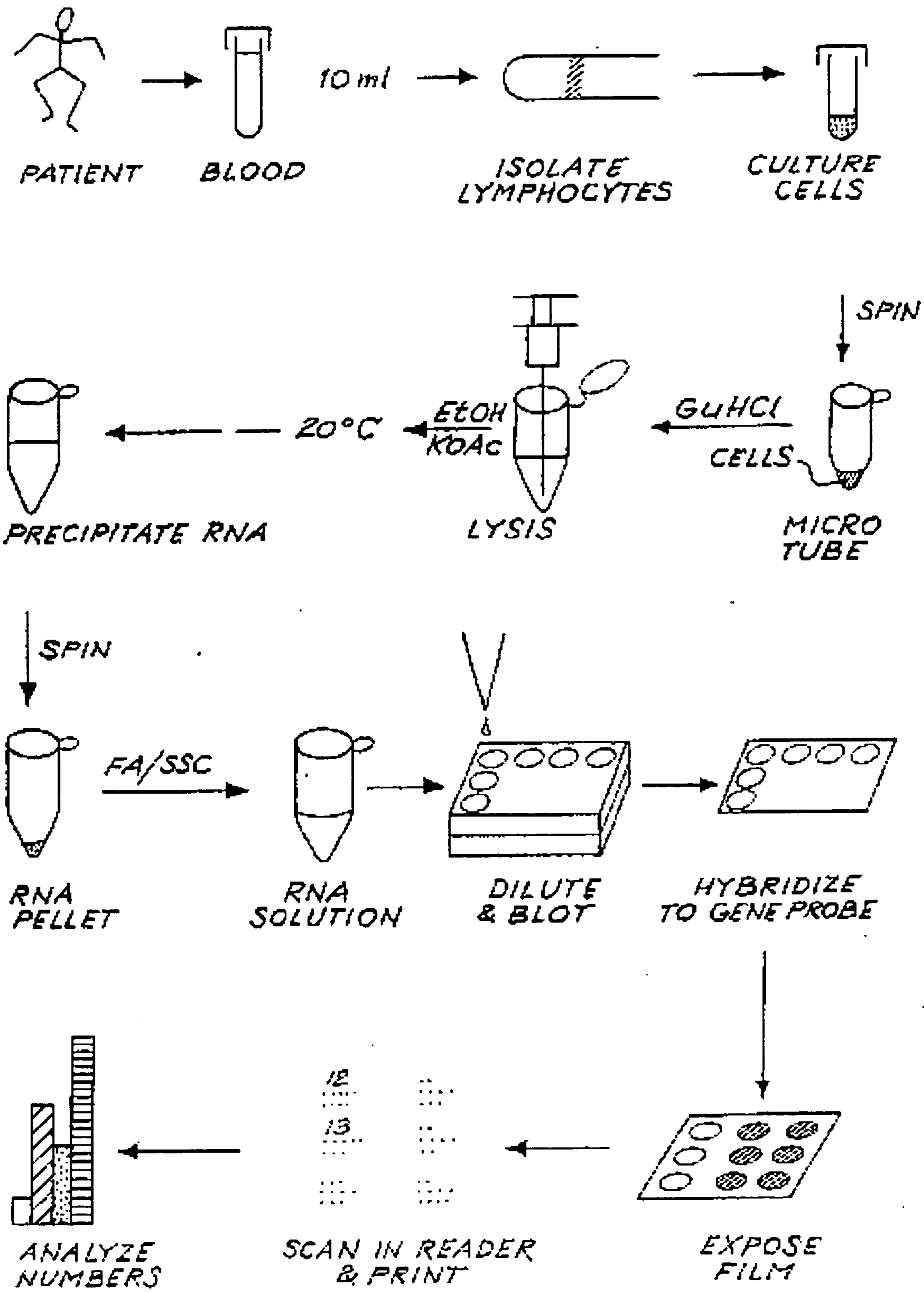
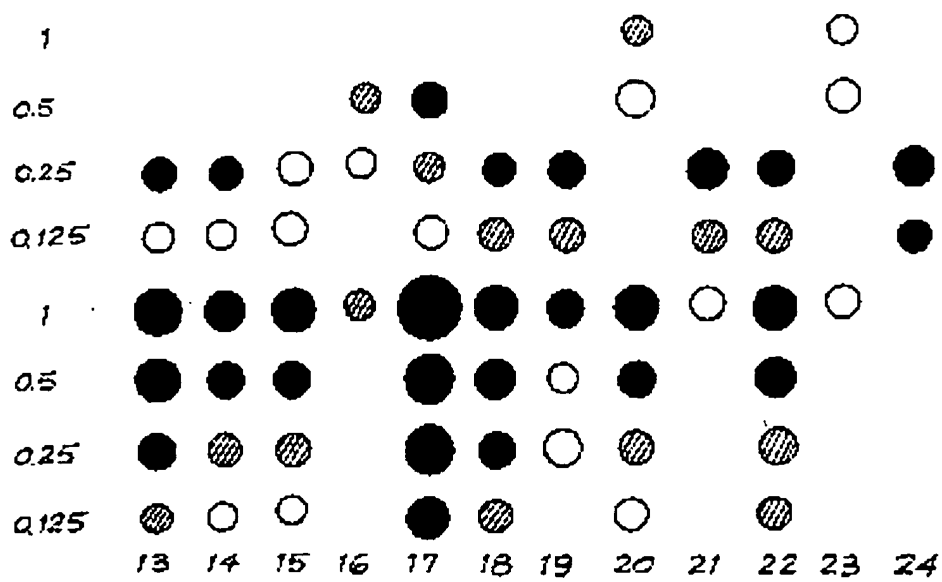

PATIENT
BLOOD
10 ml
ISOLATE LYMPHOCYTES
CULTURE CELLS
SPIN
GuHCl
CELLS
MICRO TUBE
EtOH
KOAc
LYSIS
20°C
PRECIPITATE RNA
SPIN
RNA PELLET
FA/SSC
RNA SOLUTION
DILUTE & BLOT
HYBRIDIZE TO GENE PROBE
EXPOSE FILM
12
13
SCAN IN READER & PRINT
ANALYZE NUMBERS

FIG. 1



FILM:	1	2	3	4	5	6	7	8	9	10	11	12
PHA	+	+	+	+	+	+	+	+	+	+	+	+
CHX (hr added) (20μg/ml)	-	1/2	-	-	1/2	1	-	-	1/2	1	2	-
γ-IRRADIATION	-	-	+	-	-	-	+	-	-	-	-	+
TIME of RNA EXTRACTION (hr)	1	1	1	2	2	2	2	4	4	4	4	4

	13	14	15	16	17	18	19	20	21	22	23	24
PHA	+	+	+	+	+	+	+	+	+	+	-	
CHX (hr added) (20μg/ml)	-	1/2	1	2	-	-	1/2	1	2	-	-	
γ-IRRADIATION	-	-	-	-	+	-	-	-	-	+	-	
TIME of RNA EXTRACTION (hr)	6	6	6	6	6	22	22	22	22	22	22	

SUBSTITUTE SHEET

FIG. 2

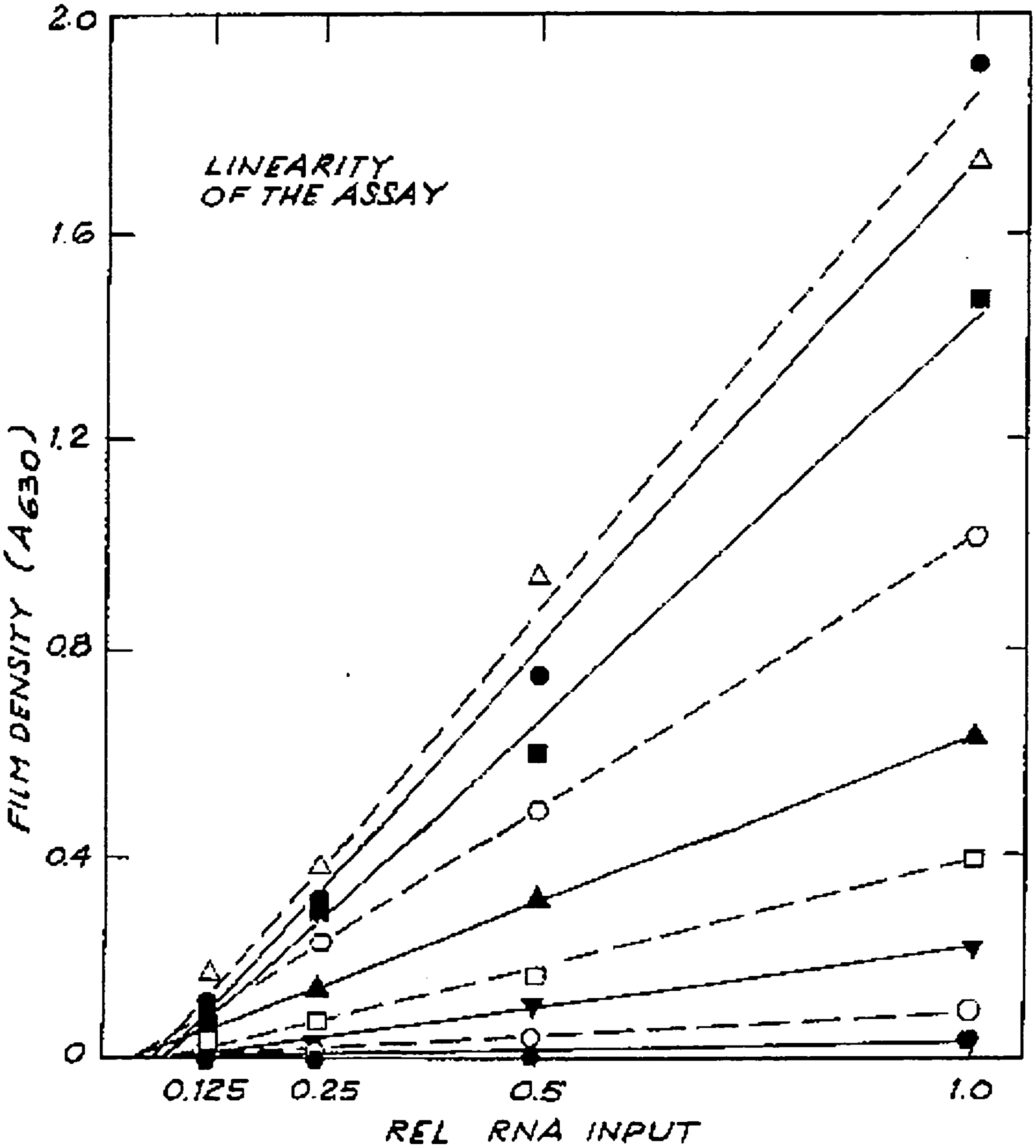
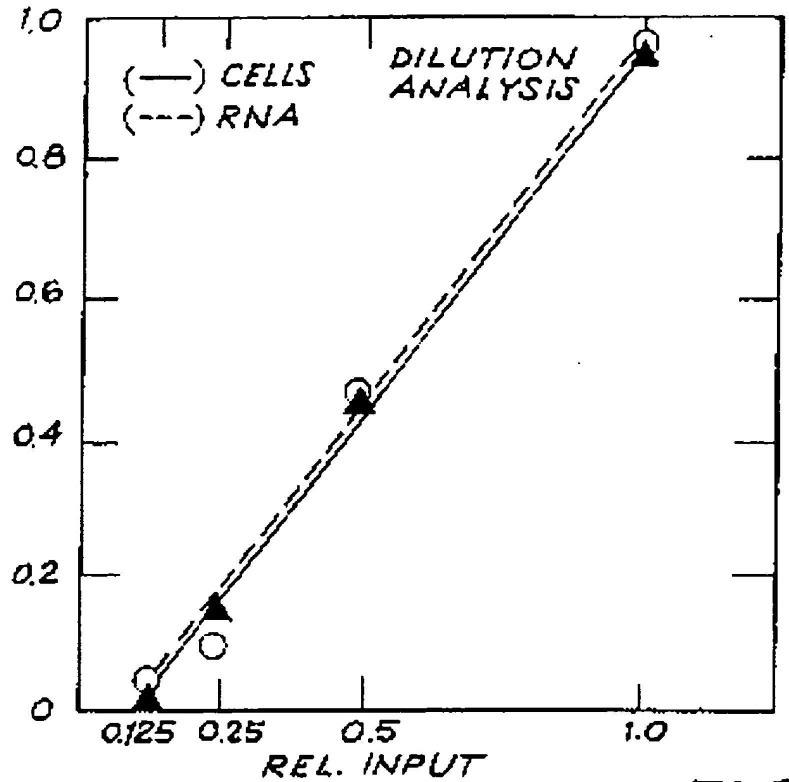
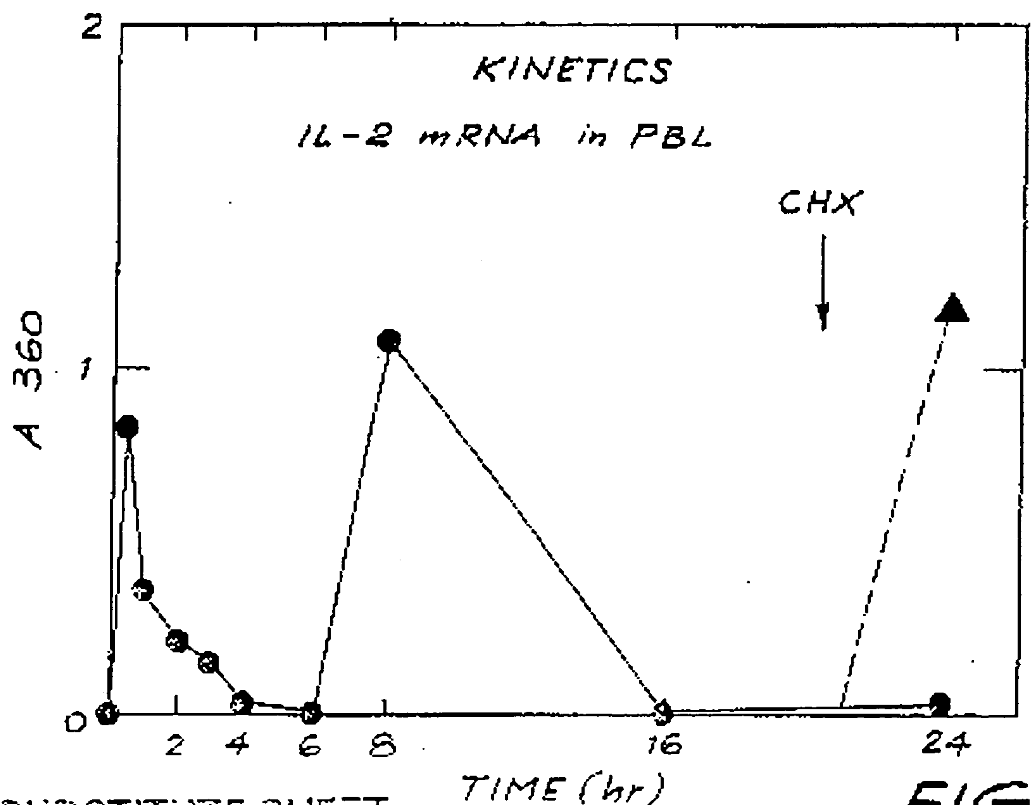

LINEARITY OF THE ASSAY
FILM DENSITY (A_{630})
2.0
1.6
1.2
0.8
0.4
0
0.125
0.25
0.5
1.0
REL RNA INPUT

FIG. 3



1.0
0.8
0.6
0.4
0.2
0
(—) CELLS
(---) RNA
DILUTION ANALYSIS
0.125
0.25
0.5
1.0
REL. INPUT

FIG. 4



2
1
0
A 360
KINETICS
IL-2 mRNA in PBL
CHX
2
4
6
8
16
24
TIME (hr)

FIG. 5

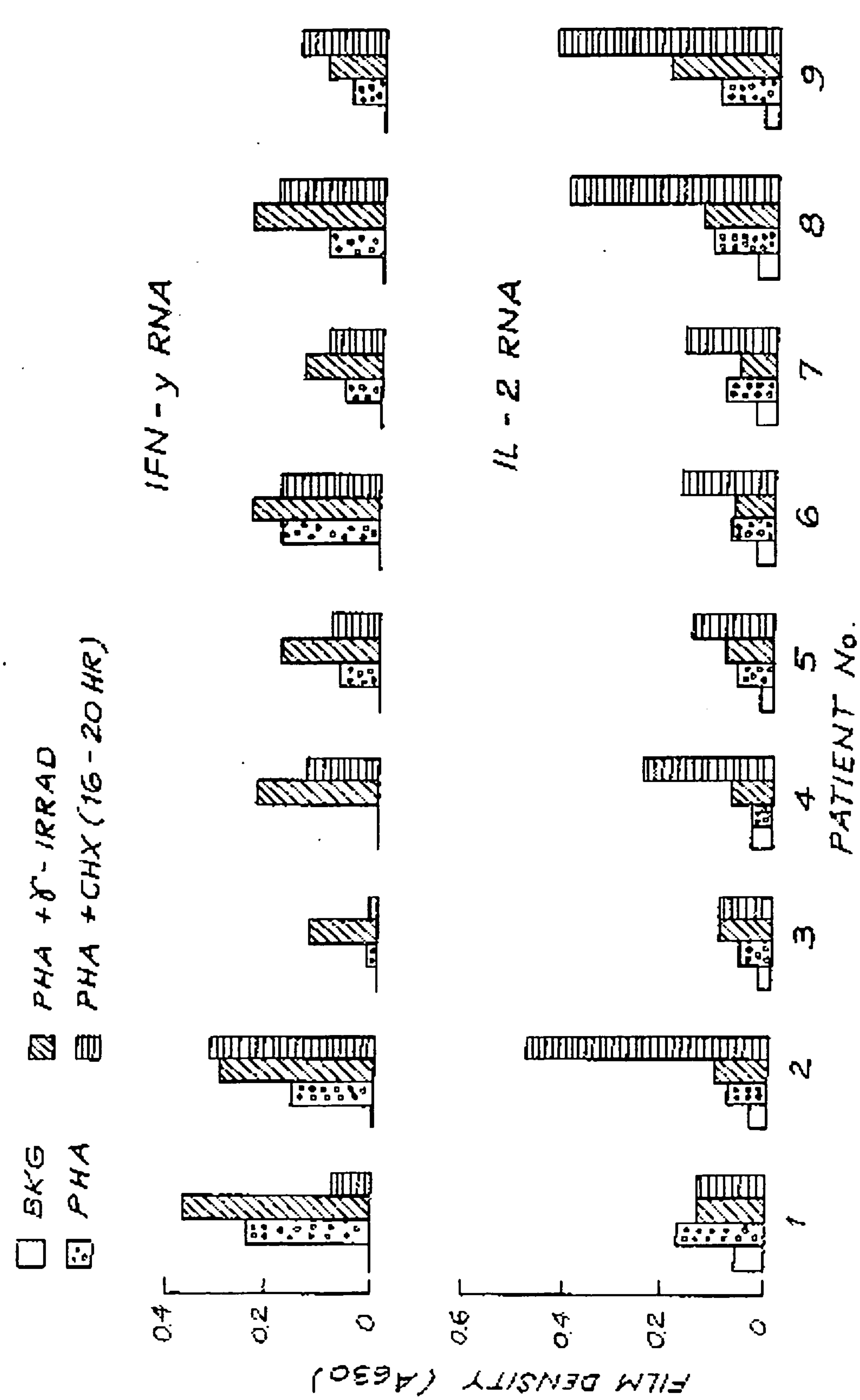

BKG
PHA
PHA + γ - IRRAD
PHA + CHX (16 - 20 HR)
IFN - γ RNA
IL - 2 RNA
0.4
0.2
0
0.6
0.4
0.2
0
FILM DENSITY (A650)
1
2
3
4
5
6
7
8
9
PATIENT No.

FIG.6

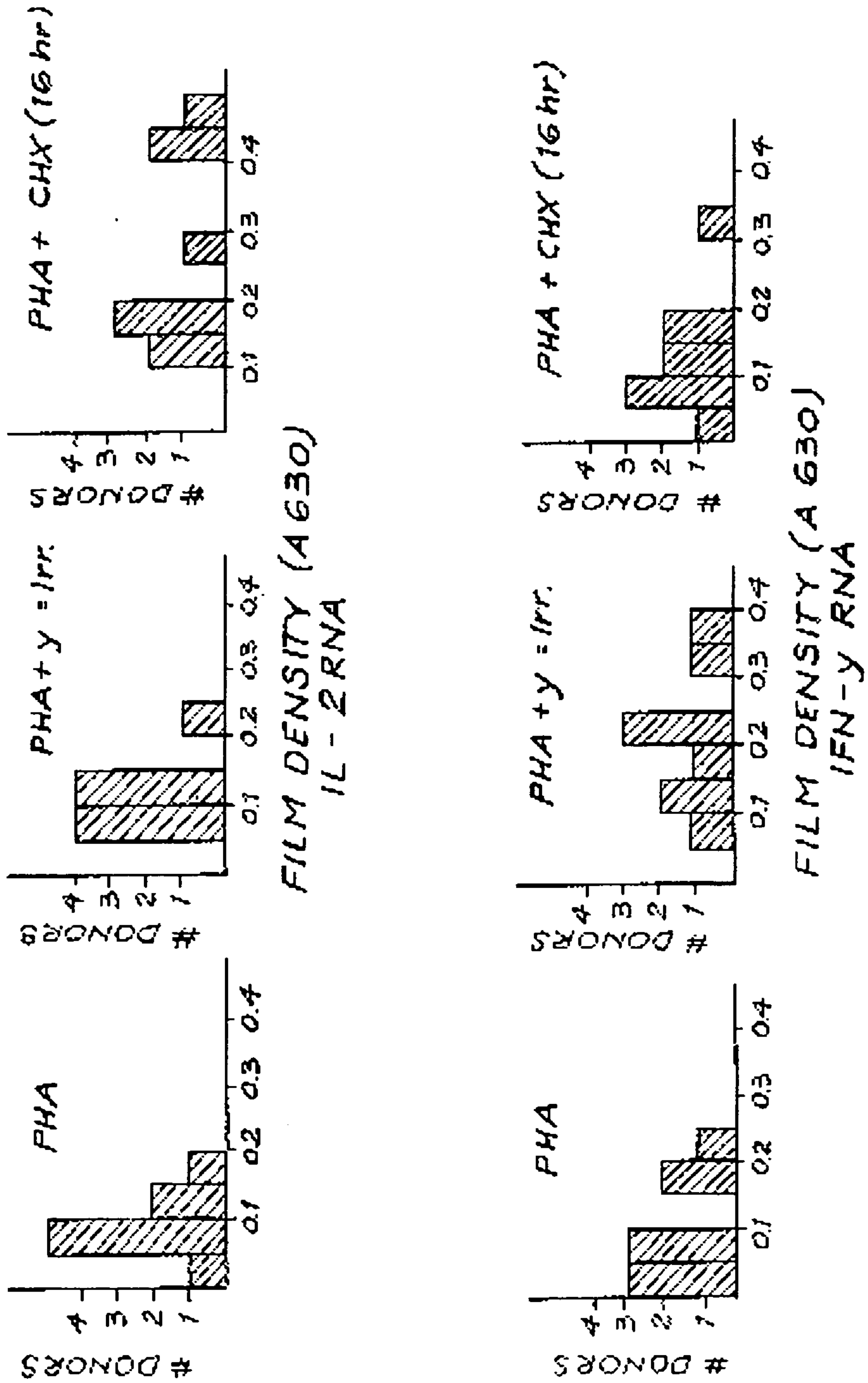

PHA
PHA + γ - Irr.
PHA + CHX (16 hr)
DONORS
4 3 2 1
0.1 0.2 0.3 0.4
FILM DENSITY (A630)
IL - 2 RNA
FILM DENSITY (A630)
IFN-γ RNA

FIG. 7